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Starvation or diabetes decreases the content but not the mRNA of 6-phosphofructo-2-kinase in rat liver

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In rat liver, the activity of 6-phosphofructo-2-kinase (PFK-2) decreases upon starvation and in diabetes. Cyclic AMP-dependent phosphorylation of the enzyme is not sufficient to account for this decrease. PFK-2 content was therefore measured by immunotitration and relative PFK-2 mRNA levels were determined by hybridization with cDNA probes. The data are compatible with a posttranscriptional mechanism of regulation that involves decreased translational efficiency of PFK-2 mRNA and(or) increased turnover of the PFK-2 protein.

mRNA; cDNA; 6-Phosphofructo-2-kinase; Fructose 2,6-bisphosphate; Diabetes; (Rat liver)

1. INTRODUCTION

Fructose 2,6-bisphosphate is an intracellular regulatory molecule that controls glycolysis in mammalian tissues by integrating hormonal and metabolic signals [1]. These signals act through 6-phosphofructo-2-kinase (EC 2.7.1.105) and fructose-2,6-bisphosphatase (EC 3.1.3.46), which respectively catalyze the synthesis and degradation of Fru-2,6-P_2. The two activities are borne by the same polypeptide chain. Although phosphorylation of the liver bifunctional enzyme can influence its activities, little is known of additional regulatory mechanisms such as transcriptional and translational control. The existence of PFK-2/FBPase-2 isozymes [1] complicates the problem.

To approach these questions, we have prepared antibody and cDNA probes for PFK-2 [2]. Rat liver was chosen as a model because PFK-2/FBPase-2 activity decreases [3–5] during starvation or diabetes. Glucagon-activated phosphorylation of the bifunctional enzyme by cAMP-dependent protein kinase does not fully account for the decrease in PFK-2 activity seen under these conditions [6]. We have therefore determined whether PFK-2 enzyme content changed in the liver of starved or diabetic rats and, if so, whether this was correlated with changes in PFK-2 mRNA levels.

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Abbreviations: Fru-2,6-P_2, fructose 2,6-bisphosphate; PFK-2, 6-phosphofructo-2-kinase; FBPase-2, fructose-2,6-bisphosphatase; PEG, polyethylene glycol

2. EXPERIMENTAL

All reagents were of the best grade commercially available. Male Wistar rats (200–250 g) on a standard Purina chow diet were rendered diabetic by a single i.v. injection of alloxan (60 mg/kg) or streptozotocin (55 mg/kg) respectively 72 h or 2 weeks before being killed. The diabetic state was assessed by a positive urine strip test (Ames) for glucose (>15 mM). This test was also positive for acetoacetate (>1.5 mM) in the alloxan-treated diabetic rats. Other rats were deprived of food 72 h before being killed. Normally fed rats served as controls. After decapitation, the liver was frozen in liquid N_2. For biochemical determinations, liver was homogenized (Potter) in 3 vols of an ice-cold solution containing 50 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 15 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, Trasylol (16 μg/ml), leupeptin (8 μg/ml), 20 mM Hepes and 1 mM phosphate, pH 7.0. PFK-2 activity

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was measured either on a supernate (10000 × g, 20 min) fractionated by 6–20% polyethylene glycol (PEG extract) or on a cytosol (105000 × g, 60 min). Unless mentioned otherwise, the assay was performed at pH 8.5 to measure the 'total' activity, which is insensitive to the phosphorylation state of the enzyme [7]. Pyruvate kinase activity, measured at 5 mM phospho*enol*pyruvate [8], and protein concentration [9] were determined as described. Total RNA was prepared from tissue homogenized in a Waring Blendor (full speed, 60 s), either by the LiCl/urea method [10] or by a modified [11] guanidinium thiocyanate [12] method. Results obtained by either technique were identical. Glycogen contaminating RNA from fed rats was pelleted by ultracentrifugation (55000 × g, 30 min) of the final ethanol precipitate of RNA resuspended in water. Poly(A)$^+$ RNA was isolated by oligo(dT) chromatography [13].

RNA-cDNA hybridization after formaldehyde/formamide denaturation was studied by the Northern [14] and dot blot [15] techniques, using rat liver PFK-2 cDNA probes labeled with [α-^{32}P]dCTP by nick translation or by the multiprime system (Amersham). Chicken β-actin and rat liver L-type pyruvate kinase cDNA probes were also used as controls in the dot blot hybridization experiments. Liver PFK-2 is a dimer of two identical 470-amino acid polypeptides. The RL2K-8 PFK-2 cDNA probe (1086 bp) corresponds to the last 304 amino acids of PFK-2 plus 174 nucleotides of the 3'-untranslated sequence [2]. Probe RL2K-22c2 corresponds to the first 90 amino acids of PFK-2 plus 173 nucleotides of the 5'-untranslated sequence. Probe RL2K-4c3 is identical to RL2K-22c2, but for the fact that it is 104 bp shorter at the 5'-end. For the dot blot mRNA assays, nitrocellulose filters were prehybridized for 5 h at 42°C in a phosphate buffer (50 mM, pH 6.8) containing 50% formamide, salmon sperm DNA (100 μg/ml), and 5 × SSC and 5 × Denhardt solutions. 1 × Denhardt is 0.02% each of bovine serum albumin, polyvinylpyrrolidone and Ficoll. 1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate. Hybridization (42°C, 16 h) was performed in a 20 mM phosphate buffer supplemented as above. Filters were then washed for 15 min at room temperature in 2 × SSC + 0.1% SDS, then for 30 min at 50°C in 0.1 × SSC + 0.1% SDS.

To obtain antibodies against native PFK-2, rabbits were immunized with PFK-2 purified [16] from chicken liver. Antiserum BCL-2 gave a positive ELISA signal at a 10^{-6} dilution with purified rat liver PFK-2. 1 μl of this antiserum precipitated 5 μU of rat liver PFK-2 activity. PFK-2 immunotitration was carried out in 0.2-ml incubations containing 100 mM KCl, 15 mM β-mercaptoethanol, 20 mM Tris, pH 8, 0.1% bovine serum albumin, 75 μl of protein A-Sepharose suspension (1/1), a fixed volume of cytosol or PEG extract and up to 50 μl of antiserum. After 30 min of agitation at room temperature, the immune complexes were precipitated (8000 × g, 4 min) and the residual PFK-2 activity was measured in the supernate.

3. RESULTS AND DISCUSSION

3.1. *Changes in PFK-2 enzyme content*

As expected from earlier work [3–6], liver PFK-2 activity in starved and in diabetic rats fell to about 50% of the values for fed rats (table 1). We therefore determined by immunotitration whether this resulted from a decreased liver content of PFK-2. As shown in fig.1A and table 1, the differences in immunoprecipitable PFK-2 content were commensurate with the differences in PFK-2 activities between fed and starved or diabetic rats.

These differences were not due to a greater efficiency of the antibody in recognizing the phosphorylated form of PFK-2. Indeed, the im-

Table 1

Liver PFK-2 activity, content, and relative mRNA levels

	Activity[a] (μU/mg protein)	Content[b] (μl)	Relative mRNA levels[c]		
			Expt A	Expt B	Expt C
Control (fed)	61 ± 5 (11)	3.39 ± 0.25 (9)	12.8 ± 1.2 (11)	13.5 ± 3.8 (3)	4.9 ± 0.5 (9)
Starved (72 h)	25 ± 2 (10)[d]	1.66 ± 0.28 (5)[d]	15.1 ± 1.4 (8)	ND	5.3 ± 1.0 (8)
Diabetic (alloxan)	37 ± 2 (5)[d]	1.55 ± 0.29 (6)[d]	17.0 ± 4.4 (5)	15.8 ± 2.5 (5)	ND
Diabetic (STZ)	24 ± 4 (6)[d]	2.45 ± 0.87 (3)	ND	ND	ND

[a] PFK-2 activity was measured on cytosol at pH 8.5 except for streptozotocin-treated rats for which the activity was measured at pH 7.1 on a PEG extract

[b] Volume of BCL-2 antiserum required to precipitate 50% of PFK-2 activity in the presence of protein A-Sepharose, calculated from logit plots of immunotitration curves such as those in fig.1A

[c] For each RNA sample, the slope of the linear relationship (see fig.2) between amount of radioactivity hybridized and quantity of RNA spotted was calculated for the PFK-2 cDNA probe and for the actin cDNA probe. The ratio ($\times 10^2$) of these slopes gives the units shown. The PFK-2 cDNA probes were as follows: Expt A, probe RL2K-8; Expt B, probe RL2K-22c2; Expt C, probe RL2K-4c3. Data in each column are not expected to match those in other columns because of differences in specific radioactivity of the probes between experimental series

[d] $p < 0.01$ vs controls by non-paired t-test

The values are means ± SE for the number of animals given in parentheses. STZ, streptozotocin; ND, not determined

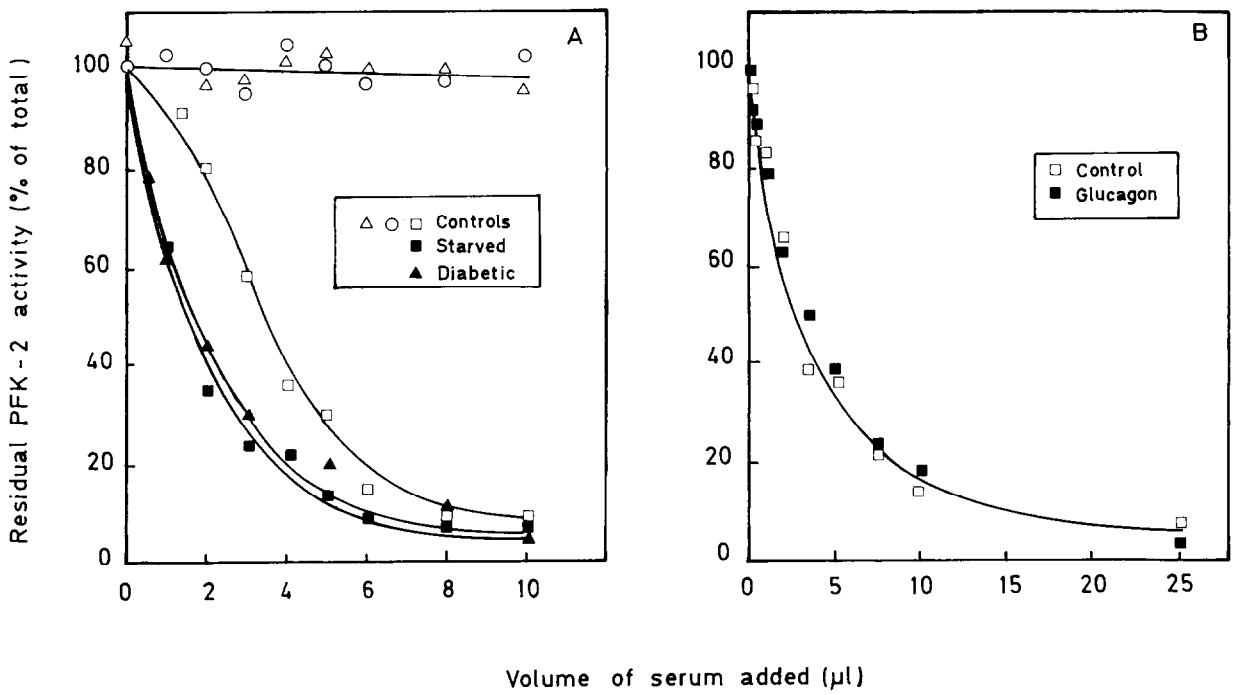

Fig.1. Immunotitration of liver PFK-2. Enzyme activity was measured at pH 8.5 after immunoprecipitation. All incubations contained 1.5 mg of cytosolic protein/ml. (A) Effect of starvation or diabetes. Total PFK-2 activity was 75 µU/mg protein in the control (open symbols), 28 µU/mg in the starved (■), and 37 µU/mg in the alloxan-diabetic (▲) rat. All incubations contained protein A-Sepharose and BCL-2 antiserum except for (○) which contained no protein A-Sepharose and for (△) in which BCL-2 antiserum was replaced by nonimmune serum. (B) Immunotitration of PFK-2 from fed rats injected (■) or not (□) in the portal vein with 0.5 ml glucagon (10^{-6} M) 5 min before being killed. PFK-2 activity was 65 and 60 at pH 8.5, and 88 and 40 µU/mg protein at pH 6.6 for control and glucagon-treated rats, respectively.

munotitration curve of phosphorylated PFK-2 (obtained from a glucagon-treated rat) was superimposable to that of a fed rat (fig.1B). Since, in the absence of protein A-Sepharose, BCL-2 antiserum did not influence liver PFK-2 activity from control (fig.1A) or treated (not shown) rats, the shift in the immunotitration curves seen in fig.1A did not result from PFK-2 activation or inhibition by antiserum [17].

3.2. *Lack of change in PFK-2 mRNA content*

The data presented above suggest that the decreased PFK-2 activity in the liver of starved and of diabetic rats results from a decreased enzyme content rather than from a change in catalytic activity, e.g. by covalent modification. One possible mechanism is a decreased steady-state level of PFK-2 mRNA. Northern blot analysis of total or poly(A)$^+$ liver RNA, hybridized with the RL2K-8 cDNA probe, revealed one band corresponding to a mRNA of about 2150 nucleotides. This suggests that liver PFK-2 mRNA precursors are very large and therefore do not migrate into the gel or do not transfer adequately onto the nitrocellulose filters.

PFK-2 mRNA was quantitated in dot blot experiments by measuring the intensity of hybridization calculated from the slope of the linear relationship between radioactivity in the blots and quantity of RNA spotted (fig.2). The data were then normalized by reference to the slope for actin mRNA in the same sample (table 1). The PFK-2 mRNA content determined with the three PFK-2 cDNA probes was the same in control and treated rats (table 1). To confirm the significance of these results, liver RNA from all experimental groups was hybridized with a cDNA probe specific for pyruvate kinase mRNA. As expected [18], the liver content of this mRNA decreased to about 50% of control values in fasted (fig.3) and in diabetic rats. Thus, had such variations in PFK-2 mRNA taken

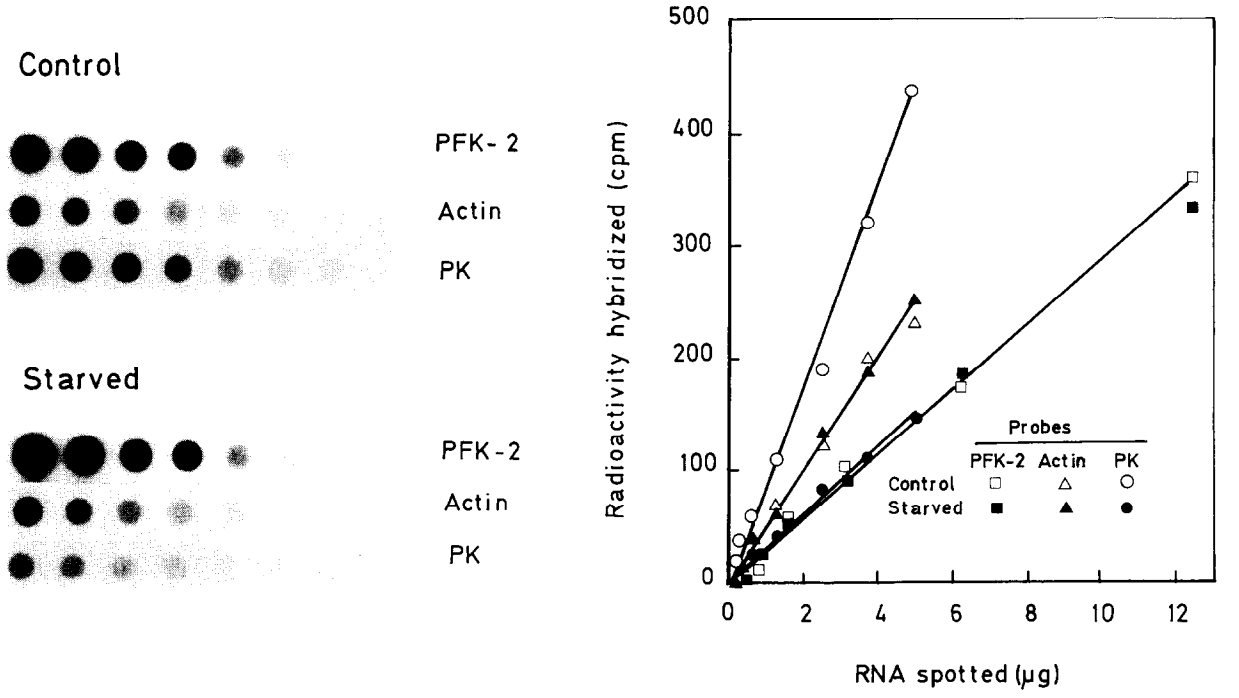

Fig.2. Dot blot hybridization of total liver mRNA with PFK-2, actin and pyruvate kinase (PK) cDNA probes. The graph was obtained by plotting the radioactivity of the spots shown.

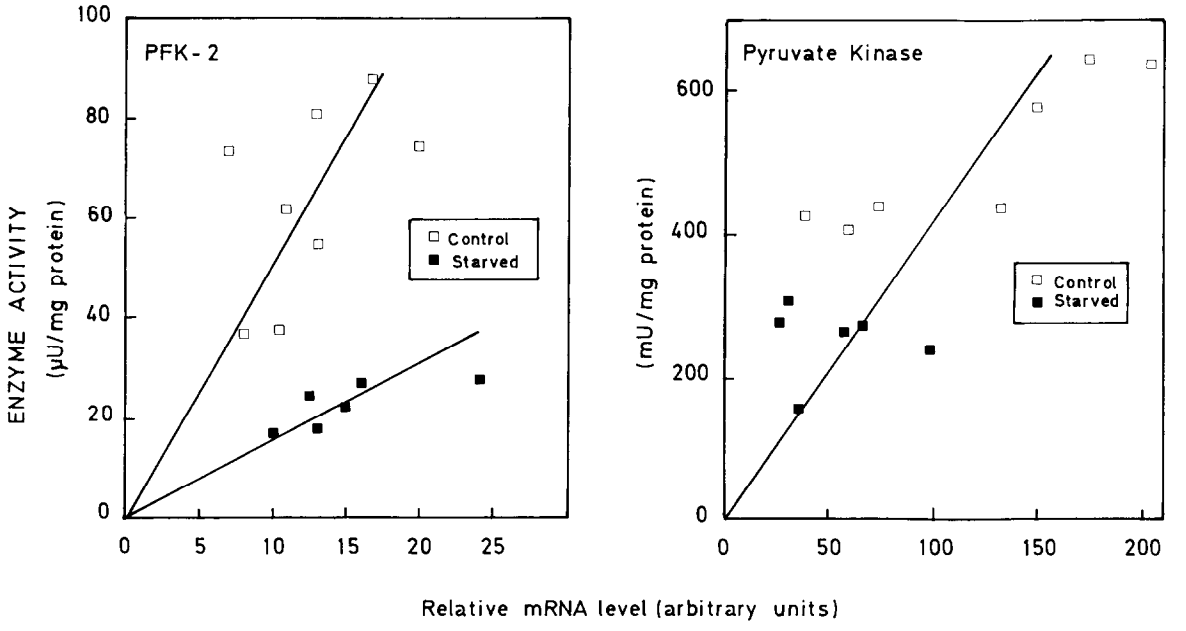

Fig.3. Effect of starvation on the relationship between enzyme activity and mRNA content in individual livers. Units of mRNA levels were calculated as in table 1. The PFK-2 cDNA probe was RL2K-8.

place, the method was sensitive enough to detect them. The data in fig.3 support this conclusion. When the liver PFK-2 mRNA content of individual rats was matched to liver PFK-2 activity of the same sample, a linear relationship was obtained, with a steeper slope for control than for starved rats. In contrast, although a linear relationship was also obtained, the slopes did not differ when pyruvate kinase mRNA content was compared to pyruvate kinase activity.

Taken together, the data suggest that the decrease in liver PFK-2 content demonstrated here in starved and in diabetic rats results from a post-transcriptional control mechanism exerted beyond mRNA synthesis or degradation. Conceivably, this could involve decreased translational efficiency [19–21] of an unchanged amount of PFK-2 mRNA, or increased turnover of the PFK-2 protein, or both.

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